Complex haplotype structure of the human GNAS gene identifies a recombination hotspot centred on a single nucleotide polymorphism widely used in association studies

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The α subunit of the heterotrimeric G protein Gs (Gsα) is involved in numerous physiological processes and is a primary determinant of cellular responses to extracellular signals. Genetic variations in the Gsα gene may play an important role in complex diseases and drug responses. To characterize the genetic diversity in this locus, we resequenced exons and flanking introns of the gene in 44 genomic samples and analysed the haplotype structure of the gene in an additional 50 African-Americans and 50 Caucasians. Significant differences in allele frequency for nearly all the genotyped single nucleotide polymorphism (SNPs) were detected between the two ethnic groups. Linkage disequilibrium (LD) analysis of this locus revealed two haplotype blocks characterized by strong LD and reduced haplotype diversity, especially in Caucasians. Between the two blocks is a narrow (approximately 3 kb) recombination hotspot centred on exons 4 and 5, and a widely used genetic marker in association studies in this region (rs7121) was in linkage equilibrium with the rest of the gene. The haplotype structure of the GNAS locus warrants reevaluation of previous association studies that used marker rs7121 and affects choice of SNP markers to be used in future studies of this locus. *Pharmacogenetics* 14:741–747 © 2004 Lippincott Williams & Wilkins

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Introduction

Cyclic AMP is a ubiquitous intracellular regulator of cell function. Primary control of cAMP levels in mammalian cells results from receptor-mediated regulation of adenylyl cyclase through the heterotrimeric G protein Gs [1–3]. The human gene for the α subunit of Gs, Gsα, is part of the GNAS locus on chromosome 20q13.3. Expression of this gene is complex and affected both by imprinting and alternative splicing. The Gsα protein itself (arising from Gsα exon 1) has four splice variants differing by inclusion (long form) or exclusion (short form) of exon 3, with both forms expressed as variants with or without a serine residue coded for at the 3′ splice donor site [4]. There are also at least three additional upstream promoters in the GNAS complex locus, each with an alternative exon 1, that splice into exon 2 of Gsα. These produce at least two other protein products, NESP55 and XLαs [5–8]. Several rare genetic diseases are known to result from null or constitutively active mutation of Gsα, or changes in imprinting and tissue-specific expression [9–13].

Given the extensive role of Gsα in cell signalling, it is not surprising that the involvement of the Gsα gene in hypertension, affective disorders, substance abuse and other complex diseases has been proposed and studied [14–20]. The most compelling genetic evidence for the involvement of the Gsα gene in a complex disease is that a silent T-C single nucleotide polymorphism (SNP) in exon 5 (ATT>ATC, Ile[131], dbSNP rs7121, Celera hcv9901536, referred to hereafter as rs7121) differs in allele frequency between hypertensive and control subjects in Caucasians (T allele 49% and 42%, respectively). This difference is most prominent in patients with hypertension who are unresponsive to β-blockers (T allele is 58.3% in poor responders, but only 37.5% in good responders) [18]. In addition, this relationship may have a complex expression pattern sensitive to other factors such as substance dependence [14,15]. Other association studies did not find any involvement of Gsα in psychiatric illnesses using the same SNP marker [16,17]. In general, association studies have not yet provided convincing evidence for a primary role of the Gsα gene in the complex diseases studied to date, even when pathophysiological and pharmacological evidence suggest involvement of the signalling pathway of which it is a major component.

Current ideas about the organization of the human

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genome suggest that genomic regions are organized into haplotype blocks separated by regions that are hotspots for recombination [21,22]. Haplotype blocks are defined by collections of SNPs that are in linkage disequilibrium (LD), meaning that they are inherited as a unit, and a SNP within a block is a reasonable marker for disease association studies characterizing the region [21,23]. Knowing the haplotype structure of a gene is an important determinant of the design of association studies to assure that any markers used cosegregate with the gene if it is related to the disease.

Although much is known about the expression of the GNAS locus, little is known about the actual inheritance pattern of the gene. Various markers have been used in association studies to test the involvement of the Gsα gene in complex diseases, with SNP rs7121 the most frequently used marker, generally assayed as a *Fok*I restriction site [14–16,18,19]. However, the assumption that this SNP is in LD with potential contributing mutations elsewhere in the gene has never been tested. Here, we describe the haplotype structure of the Gsα region of GNAS, and find that it contains two primary haplotype blocks separated by an intervening recombination hot spot of approximately 3 kb centred on exons 4 and 5 of the gene. This structure indicates that past studies of the association of this gene with human disease may have missed potential associations because they used a common SNP that is in linkage equilibrium with the rest of the gene.

Materials and methods

DNA samples

DNA samples were obtained from National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository (Coriell, http://locus.umdnj.edu/nigms/). Resequencing of the Gsα gene used a subset (44 subjects) from the Coriell Polymorphism Discovery Resource (M44PDR) composed of 27% of European Americans, 27% Asian-Americans, 27% African-Americans, 13% Mexican-Americans and 6% Native Americans [24]. Fifty African-Americans (HD50AA) and 50 Caucasians (HD50CAU) from the Coriell Human Variation Panels were used for genotyping of common SNPs for haplotype structure analysis.

Resequencing of the GNAS gene locus

Polymerase chain reaction (PCR) used specific primer sets with reagents from Qiagen (Taq PCR core kit, catalogue #: 201223; Qiagen, Valencia, California, USA) to amplify Gsα exons and intron/exon junctions in 44 subjects from the Coriell Polymorphism Discovery Resource. PCR of exons 2–13 used a melting temperature of 95°C for 30 s, annealing temperature of 55°C for 30 s and extension temperature of 72°C for 40 s. Amplification of exon 1 used an annealing temperature of 62°C for two cycles and then 55°C for 35 cycles, and required 1 × Q solution from the Qiagen kit in the final reaction. All the reactions used 100–150 ng of genomic DNA and were amplified for 35–40 cycles. PCR products were separated by 2% agarose gel, visualized under ultraviolet light, purified using Qiagen QIAquick PCR Purification Kit (Qiagen, Cat no. 28106) and sequenced on an ABI Model 377 Automated Sequencer (Applied Biosystems Inc., Foster City, California, USA) through the MUSC Biotechnology Core Facility.

Genotyping by sequencing, restriction digestion and TaqMan RT-PCR

Genotyping of the common SNPs in 50 African-Americans and 50 Caucasians from Coriell Human Variation Panels used a combination of sequencing on an ABI 377, restriction digestion for the *Fok*I site [18] and TaqMan real time-PCR technology [25], with both commercial (Assay-on-Demand) and custom designed (Assay-by-Design) SNP detection kits (Applied Biosystems Inc.). For sequencing and restriction digestion, DNA was prepared as above. TaqMan assays used 1–10 ng genomic DNA with commercial reagents (Applied Biosystems Inc.) on either an ABI 7000 or ABI 7300 RT-PCR. All three assays were in agreement for the *Fok*I site.

Calculation of LD and statistical analysis

Linkage disequilibrium (LD) was analysed using the normalized disequilibrium coefficient $|D'|$ [26], and the correlation coefficient r and r^2 using informative genotypes [27]. These parameters characterize the strength of association of two SNPs and indicate greater association (coinheritance) when their values are closer to 1. For two biallelic loci, L1 and L2, each with alleles 1 and 2,

$$D = f_{11}f_{22} - f_{12}f_{21}$$

where f_{ij} is the frequency of haplotype combinations between L1 ($i = 1,2$) and L2 ($j = 1,2$). The frequencies of the two alleles in L1 are p_1 and p_2, with $p_2 = 1 - p_1$; and those in L2 are q_1 and q_2, with $q_2 = 1 - q_1$. Normalized D, $|D'|$, comparing LD regardless of allele frequency is:

$$|D'| = |D|/D_{max}$$

where $D_{max} = \min(p_1q_2; p_2q_1)$ when D is positive, and $D_{max} = \min(p_1q_1; p_2q_2)$ when D is negative. $|D'|$ is 1 when two sites are in complete LD (only three of the four haplotype combinations exist), but it is also 1 when both minor allele frequencies are low, and the minor alleles tend not to be seen together on the same chromosome, leading to a $|D'|$ of 1 but with very little power to detect recombination [26]. Therefore, the correlation coefficient was calculated as:

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$$r^2 = D^2/(p_1 p_2 q_1 q_2)$$

r^2 assumes a value of 1 when two sites are in absolute LD (only two of the four possible haplotype combinations exist) and there is a simple inverse relationship between r^2 and the sample size required to reach the same statistical significance when the mutation site is not directly genotyped [28]. As a result, r^2 is, arguably, a better parameter than D' for use in designing association studies.

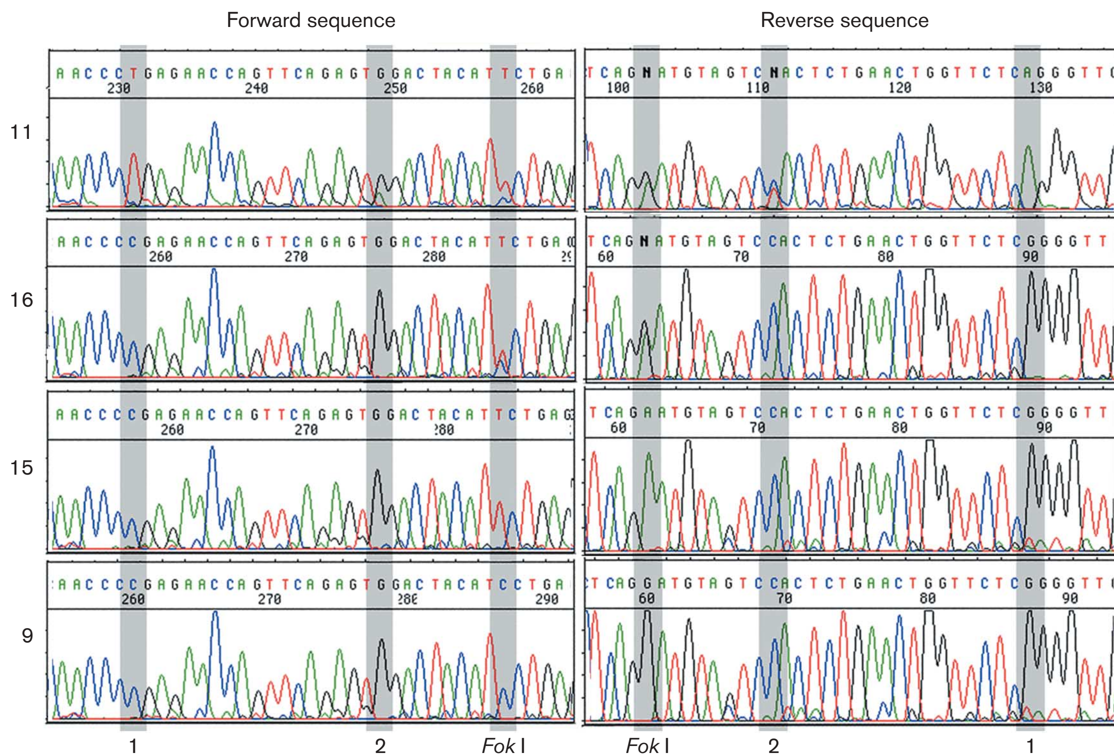
Haplotype prediction

Haplotypes and their frequencies in each ethnic group were estimated from genotype data via both the phase reconstruction method (PHASE) [29,30] and the maximum likelihood method from an expectation maximization algorithm under the assumption of Hardy–Weinberg equilibrium and random mating in the population [31]. Both methods produced comparable estimates of the major haplotypes and the PHASE results are reported.

Results and discussion

These studies aimed to characterize the prevalent heterogeneity and gene structure of the Gsα region of the GNAS locus. Resequencing of all of the coding regions and surrounding intron junctions for the Gsα region in 44 Polymorphism Discovery Resource samples did not reveal any non-synonymous mutations. However, three new polymorphisms were found: two of them in exon 5, and the other in the adjoining intron 5. Interestingly, all of these were found in one individual, who was homozygous for two of the three SNPs (Fig. 1). These results suggest that this individual came from an isolated ethnic group in whom these polymorphisms exist in high frequency. It is important to note that the Coriell sample set, which was designed to maximize polymorphism discovery [24], represents populations that are diverse enough to contain such samples. Our

Fig. 1



Genetic variation of exon 5 of Gsα in ABI sequencing records. Exons 4 and 5 of Gsα were amplified by polymerase chain reaction from DNA samples from the Coriell Polymorphism Discovery Resource and sequenced on an ABI 377. The region around exon 5 containing the commonly used *Fok*I restriction site is shown for the Coriell subject numbers shown on the left. Both forward and reverse sequences are shown. Regions of genetic variation are highlighted in grey. Subject 11 was unique among the Coriell dataset in having three single nucleotide polymorphisms (SNPs) in this region (and nowhere else in the Gsα gene), for which this individual is homozygous for two of these. The panel shown contains the sequence around the *Fok*I site and two of the three unique SNPs in subject 11. The third one is just 3′ of this region. Subjects 11 and 16 are heterozygous at the *Fok*I site, whereas subjects 9 and 15 are homozygous C and T, respectively, on the forward strand. At sites 1 and 2, subject 11 differs from all others in the study. At site 1, this individual is homozygous T, whereas all others are homozygous C; and at site 2 heterozygous A/G, whereas all others are homozygous G, on the forward strand.

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results also appear to indicate that the region around exon 5 is unique within the Gsα gene and may be a region of high genetic variability. These three SNPs were all within 140 bp of one another and in or near exon 5. Other than this small region, this individual had 1100 nucleotides of DNA sequence throughout GNAS coding and intron/exon junctions that are identical with that of the other 43 individuals in the Coriell dataset.

Because haplotypes are more informative in associating genotypes with phenotypes, these studies also aimed to determine the haplotype structure of the Gsα component of the GNAS locus. We did this by genotyping 10 common polymorphic sites in this gene in samples from Coriell Human Variation Panels (50 African-Americans, 50 Caucasians) because they are ethnically identifiable. The 10 SNPs used were identified in the Celera database or by our own characterization of this region (Table 1). All the SNPs were found in both populations, although with ethnic allele frequency differences were as great as 30% (Table 1). All but one of the SNPs had minor allele frequencies of 10% or greater in African-Americans, and 21% or greater in Caucasians. Thus, most of these SNPs are potentially useful markers in future association studies involving Gsα. No significant deviation from Hardy–Weinberg equilibrium was detected for any of the sites genotyped (data not shown).

Linkage disequilibrium among SNP sites was analysed by calculating the pairwise LD coefficient $|D'|$, and the LD correlation coefficient r^2 between SNPs (Fig. 2). Pairwise analysis of LD among the 10 SNPs genotyped revealed that the five SNPs 5′ to rs7121 are in strong LD (with pair-wise $r^2 = 0.45–0.93$, $|D'| = 0.83–1$, $P < 0.0001$ by chi-squared in Caucasians). These data suggest the existence of a haplotype block spanning more than 20 kb from 10 kb upstream of exon 1 of Gsα to the middle of intron 3. Similarly, three of the four SNPs 3′ to rs7121, from intron 6 to intron 12, are in strong LD (with $r^2 = 0.69–0.92$, $|D'| = 0.84–1$), suggesting the presence of a second haplotype block. By contrast, one SNP (SNP 9 in Fig. 2, hcv7611069) showed high $|D'|$ values with the other three SNPs, but very low r^2 values (discussed below). From the data in Fig. 2, it is conspicuous that none of the SNPs on one side of rs7121 are substantially correlated with any of the SNPs on the other side, even for SNPs less than 2 kb on either side of rs7121. The haplotype block patterns were more obvious in Caucasians than in African-Americans. This would be expected from past studies indicating greater genetic diversity in populations of African descent [22,32,33], and current ideas about the evaluation of haplotype blocks suggest that blocks are more prominent and more accurately evaluated in European populations [34]. Nevertheless, evidence of the major blocks was still present in African-Americans and, once again, the most striking result was the lack of correlation of any of the nine SNPs with rs7121, or any SNP pairs across rs7121.

Our results show that rs7121, the most frequently used marker in association studies of Gsα, does not associate with any other SNPs typed in this gene, as reflected by low $|D'|$ and r^2 values in both African-American ($r^2 \leqslant 0.04$, $|D'| \leqslant 0.41$) and Caucasian ($r^2 \leqslant 0.06$, $|D'| \leqslant 0.4$) populations (Fig. 2). Both homologous recombination and recurrent mutation can cause loss of LD. Although recurrent mutation is considered rare, analysis of variation in the lipoprotein lipase (LPL) gene suggested that multiple mutational hits at certain sites can occur [35]. However, it is unlikely that recurrent mutation at the rs7121 site would explain our observations because that would not disrupt LD between SNPs in the 5′ block and those in the 3′ block. In our data, there is nearly complete disruption of LD between all sites on either side of rs7121 (Fig. 2). The

Table 1 Ethnic-based allele frequencies of single nucleotide polymorphisms (SNPs) in the human Gsα gene

	African-American					Caucasian			
SNPs	Minor allele frequency (%)	Homozygous on minor allele (%)	Homozygous on major allele (%)	Heterozygous (%)	Allele frequency differences (%)	Minor allele frequency (%)[a]	Homozygous on minor allele (%)	Homozygous on major allele (%)	Heterozygous (%)
(1) 9338497	49	22	24	54	11	38	20	44	36
(2) 9338486	49	22	24	54	7	42	22	38	40
(3) 9901568	27	8	54	38	25	52	20	38	42
(4) 1407040	26	6	54	40	14	40	20	40	40
(5) 3Br[b]	49	18	20	62	5	44	20	32	48
(6) 9901536 (rs7121)	25	8	58	34	28	53	28	22	50
(7) 3263668	11	–	78	22	30	41	22	40	38
(8) 2174654	8	–	84	16	29	37	16	42	42
(9) 7611069	34	10	42	48	13	21	8	66	26
(10) 1081302	10	–	80	20	27	37	14	40	46

[a]Minor allele designates alleles less frequent in African-American population. The same allele could be the major allele in Caucasian population. [b]Single nucleotide polymorphism identified in intron 3 in these studies 773 bp upstream of exon4 TTTTTTTGAC[A/G]GAGTCTCACT. –, Genotypes that were not detected in our dataset.

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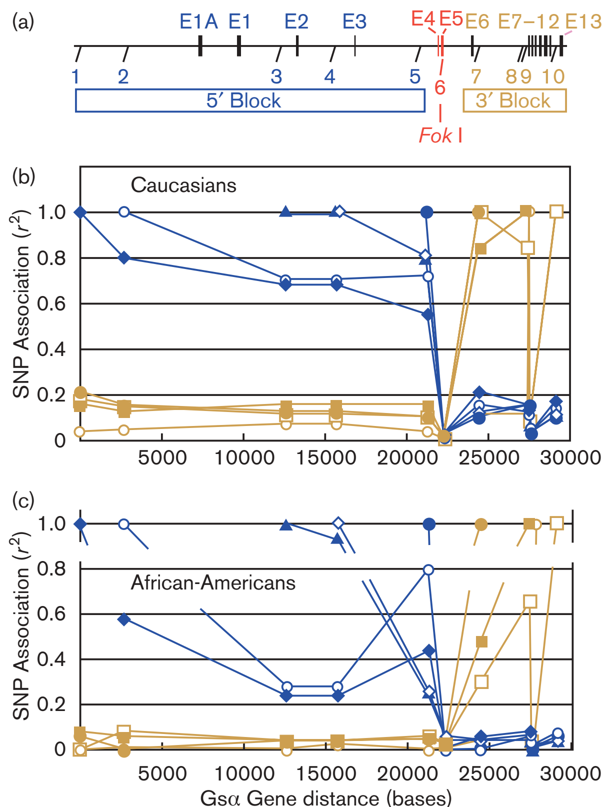
Fig. 2



Linkage disequilibrium analysis of 10 single nucleotide polymorphisms (SNPs) in Gsα gene. (a) SNP distribution on the Gsα gene and relationship to coding exons. SNP designation was according to Celera database hcv# (numbers in the figure refer to the associated hcv#; symbol designations in parentheses are used in the rest of the figure): 1, hcv9338497 (filled blue diamond); 2, hcv9338486 (open blue circle); 3, hcv9901568 (filled blue triangle); 4, hcv9901560 (open blue diamond); 5, 3br (filled circle); 6, hcv9901536 (rs7121); 7, hcv3263668 (filled tan circle); 8, hcv2174654 (filled tan square); 9, hcv7611069 (open tan circle); 10, hcv1081302 (open tan square). Also indicated is the proposed haplotype structure of this portion of the gene and the position of the proposed recombination hotspot around in exon 5. (b) Data for 50 Caucasian subjects from the Coriell Human Variation Panel. Blue symbols and lines indicate pairwise r^2 values of SNPs in the 5′ block with themselves (by definition equal to 1) and with all SNPs 3′ to them. Each SNP is indicated with a different symbol, given above. Tan symbols and lines indicate pairwise r^2 values for SNPs in the 3′ block with themselves and with all SNPs 5′ to them. A clear drop of r^2 value across marker rs7121 in exon 5 emphasized the lack of linkage disequilibrium of this site with all other sites, and of SNPs on one side to all other SNPs on the other side of rs7121. This indicates the random association of SNPs in the 5′ block with those in the 3′ block. (c) Data for 50 African-American subjects from the Coriell Human Variation Panel. Note that the *y*-axis is broken and that pair-wise r^2 values are, in general, lower for these samples than for the Caucasian samples. The reasons for this are discussed in the text. In spite of this, the striking pattern denoting lack of association of SNPs on either side of rs7121 remains very clear.

fact that there is no strong LD detected between any pairs spanning rs7121 strongly suggests that recombination around this site was the major factor causing the observed LD pattern of the gene. Computational analysis by statistical estimation of recombination rates from genotypic data using PHASE [29,36] or HotSpotter [37] also predicted higher recombination rates in the region around rs7121 in both populations (data not shown). All of these results indicate that exon 5 of Gsα is indeed located in a region of high recombination rate. Recombination hotspots have also been associated with increased mutation rate or increased genetic variability [38,39]. The data in Fig. 1 may support this idea because they show that the region around exon 5 is unique or different among Gsα coding regions and intron/exon junctions in a population of 44 genetically diverse individuals. This variation was not just chance sampling of an individual with a rare genotype because this individual was homozygous for two of three SNPs found in this region. Thus, our data indicate the existence of a narrow, 2–4 kb, recombination hotspot centred on a coding exon of the GNAS gene containing the most commonly used marker for studies of its inheritance. This is a relatively rare event because a genome-wide survey of recombination sites indicates that they occur preferentially outside genes [34]. These findings have implications both for the structure of this gene and for studies evaluating its relationship to disease and treatment.

We constructed separate haplotype maps for the 5′ and 3′ blocks of the Gsα gene [40] using the phase reconstruction method that analyses diploid genotype data [29] (Table 2). Just as large SNP allele frequency differences were found between ethnic populations (Table 1), there were large haplotype frequency differences as well (Table 2). In Caucasians, the two most frequent haplotypes accounted for 89% of the chromosomes, and other haplotypes had predicted frequencies of less than 5%. In African-Americans, five haplotypes had predicted frequencies higher than 5%, and the two major haplotypes represented only 63% of total chromosomes in the population. Interestingly, the two most frequent haplotypes in either ethnic group contained completely mismatched SNPs for the 5′ block. High frequency, almost completely mismatching SNP haplotype pairs is a common observation in human populations and has been designated as ‘yin yang haplotypes’ [41]. This dominance of major haplotypes in a chromosomal region (haplotype blocks) is extremely useful in association studies. The fact that regions both upstream and downstream of the exon 5 marker in the Gsα gene are in haplotype blocks, and the derivation of hierarchical structure of the haplotypes, will make it possible to choose only a few SNPs to represent the majority of the haplotypes of the gene in a given population.

The 3′ haplotype block identified has a complex structure. One of the SNPs in the block (hcv7611069 or SNP 9 in Fig. 2) had a very low r^2 value with the other three sites in the block, even though it demonstrated strong LD by $|D'|$ ($|D'| = 1$ with hcv2174654 in both

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Table 2 Predicted haplotypes and estimated population frequencies calculated by PHASE using genotypic data for the single nucleotide polymorphisms (SNPs) 5′ and 3′ to rs7121

SNPs 5′ to rs7121			SNPs 3′ to rs7121		
Haplotype sequence (5′)[a]	Frequency in African-Americans (%)	Frequency in Caucasians (%)	Haplotype sequence (3′)[b]	Frequency in African-Americans (%)	Frequency in Caucasians (%)
AGCTG	38.1	53	GATC	55.0	37.9
TATCA	23.0	36	GACC	32.2	19.5
TACTA	10.6	–	AGTT	8.0	34.9
AACTA	6.8	–	AATC	2.5	4.1
TACTG	5.9	2	GACT	1.4	–
TGCTG	4.9	–	AGTC		1.0
TGCTA	3.6	–	GGTT	–	1.1
AGCTA	2.3	4	Other[c]	0.5	1.5
AATCA	1.5	4			
AGTTG	0.9	1			
AACTG	0.8	–			
TGTCA	0.7	–			
Other[c]	0.9	–			

[a]Sequences represent genotypes of SNPs in the 5′ block (in order): hcv9338497, hcv9338486, hcv9901568, hcv1407040 and 3Br. [b]Sequences represent genotypes of SNPs in the 3′ block (in order): hcv3263668, hcv2174654, hcv7611069 and hcv1081302. [c]Haplotypes that have estimated population frequency of 0.5% or less.

Caucasians and African-Americans) and is only 140 bp downstream of another SNP (SNP 8 in Fig. 2) that is well associated with the rest of the block. Such isolated SNPs, falling in regions of otherwise well linked SNPs, is not an uncommon phenomenon in the human genome [42]. However, this is probably not caused by recombination because that would break the LD between other SNPs in the 3′ region. High $|D'|$ values and low r^2 values between this site and other SNPs in this region, even in Caucasians where minor allele frequencies are relatively high, suggests that this is explained by haplotype allele frequency rather than by recombination, or by gene conversion [43], which appears to have a greater role on the inheritance of individual SNPs than once recognized. Most importantly, whatever the explanation of the occurrence of low r^2 between this site and others in the 3′ block, it does not affect the conclusion about the recombination hotspot around rs7121.

Our findings emphasize the importance of using haplotypes instead of single SNPs as markers in association studies. The widely used SNP marker rs7121 in exon 5 of the GNAS gene is located in a recombination hotspot that is in linkage equilibrium with markers both upstream and downstream. Previous association studies using SNP rs7121 or other single markers of the Gsα gene found negative, weak, or controversial results about the involvement of the gene in susceptibility to hypertension, bipolar disorders, major depression and drug responses [14–20]. The fact that SNP rs7121 is in linkage equilibrium with markers as close as 1–2 kb, both upstream and downstream, suggests that any negative conclusions of these previous studies may have to be re-examined. Because there is a recombination hotspot in the middle of the gene, markers located in one of the two haplotype blocks would only represent one part of the gene. Consequently, the haplotype characterization of the gene reported here both indicates the need for comprehensive genotyping based on haplotypes of the gene in future association studies involving Gsα, and indicates minimum sets of SNPs for this characterization. The association of the rs7121 site in GNAS exon 5 with hypertension and, in particular, a subset of patients that are unresponsive to β-blockers [14,15,18], although not strong, is interesting given the results reported here. Our results indicate that the hotspot itself may have a direct relationship to any previous association of this site with hypertension because it is in equilibrium with all regions of the gene both upstream and downstream.

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